Transcription of the Herpes Simplex Virus Latency-Associated Transcript Promotes the Formation of Facultative Heterochromatin on Lytic Promoters

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An important question in virology is the mechanism(s) by which persistent viruses such as the herpesviruses and human immunodeficiency virus (HIV) establish a latent infection in specific types of cells. In the case of herpesviruses, herpes simplex virus (HSV) infection of epithelial cells results in a lytic infection, whereas latent infection is established in sensory neurons. Recent studies have shown the importance of chromatin structure in the regulation of latent infection for both HSV and HIV. For HSV, we have shown previously that the viral latency-associated transcript (LAT) promotes lytic gene silencing and the association of one heterochromatin marker, dimethylation of lysine 9 on histone H3 (H3K9me2), with viral lytic genes. In this study, we further defined the structure of latent viral chromatin by examining the heterochromatin markers on histones associated with the HSV latent genome. We detected the H3K9me2, H3K9me3, and H3K27me3 modifications, which is indicative of facultative heterochromatin, exhibiting the highest enrichment on all viral promoters tested. A modification associated with cellular centromeric heterochromatin, H4K20me3, was not detected. A mutant virus containing a 1.8-kbp deletion within the LAT region showed reduced levels of the facultative heterochromatin marker (H3K27me5) along with H3K9me3 during latency, whereas a viral mutant defective for the LAT promoter showed a specific reduction in H3K27me3. Cellular long, noncoding RNAs induce facultative heterochromatin, and this study shows that transcription of a viral noncoding RNA can also induce facultative heterochromatin to promote lytic gene silencing during latency.

Reservoirs of persisting virus in the form of latent infection present a challenge to virus eradication due to their resistance to current antiviral therapies. In the case of human immunodeficiency virus (HIV), deactivation of an infected T cell results in silencing of viral gene expression and entry into a quiescent state (56). Herpes simplex virus 1 and 2 (HSV-1 and -2) establish a latent infection within sensory ganglia, in which the lytic genes are silenced, and the only viral gene products detected at high levels are a family of noncoding RNAs collectively referred to as the latency-associated transcripts (LATs) (25, 46). The mechanism(s) by which viral genes are silenced during latent infection and how silencing is overcome to result in virus reactivation are not clear. Given that viral reactivation can result in recurrent infection and transmission to a new host, a greater understanding of the molecular mechanisms that regulate gene expression during latency could potentially yield new therapies.

A proposed mechanism of lytic gene silencing for both HIV and HSV is the association of heterochromatin with the viral genomes (18, 23, 33). In the case of HSV-1, high levels of histones accumulate on lytic promoters during the establishment of latency (14, 55), and the histones present are hypoacetylated (27) and enriched for the heterochromatin marker histone H3 dimethyl-lysine 9 (H3K9me2) (55). The mechanism(s) by which the chromatin structure on lytic promoters is regulated within neurons is not understood, although key viral candidates are the LATs. The most abundant LAT species are 2-kb and 1.5-kb stable introns (19, 44, 54), which are processed from a primary 8.3-kb LAT transcript that is expressed from a neuron-specific promoter/enhancer (2, 4, 30, 61). Micro-RNAs have also been detected within the LAT region (13, 49, 53). The LATs have been found to promote the downregulation of lytic gene expression (9, 20, 31), and a recent study identified a role for the LATs in promoting the H3K9me2 modification on lytic promoters (55), suggesting a role for the LATs in the assembly of heterochromatin on lytic promoters.

To determine the mechanism(s) by which LATs promote heterochromatin formation on lytic promoters, it is important to determine the nature of the heterochromatin on the latent genome. Heterochromatin has been divided into two general types: constitutive and facultative (cHC and fHC, respectively). cHC is used to define regions of heterochromatin that are consistently found in all cell types, whereas fHC is transcriptionally silent but has the capacity to convert into euchromatin to allow active gene expression (52). fHC has been distinguished from cHC on the basis of differential methylation patterns on the core histone proteins. For example, regions of fHC generally contain high levels of H3K9me2, H3K27me3, and H4K20me, whereas cHC contains H3K9me2, H3K9me3, and H4K20me (52). The mechanism by which these different types of modifications are targeted to specific genomic regions is not fully understood, although there is evidence that non-coding RNAs play a role in the targeting of fHC and cHC (59).
To determine the mechanism by which heterochromatin associates with the HSV-1 genome during latency, it was necessary to further define the structure of latent viral chromatin. By performing chromatin immunoprecipitations (ChIPs) on latently infected ganglia, we have found high levels of H3K27me3, a hallmark of cellular fHC, on the latent viral genome. Furthermore, by carrying out infections with viruses with mutations in the LAT region of the genome, we have identified a role for transcription of the LATs in promoting fHC formation on lytic promoters.

**MATERIALS AND METHODS**

**Cells and viruses.** Vero cells were maintained as described previously (11). The wild-type (WT) strain of HSV-1 (KOS) used in this study was grown and titrated as described previously (24, 28). The LAT gene deletion virus KlLAT, the repaired virus KlLAT, and their propagation and titration have been described elsewhere (20). The KOS\textsubscript{Pst}LAT mutant virus contains a 202-bp PstI-PstI deletion encompassing the core LAT promoter, which is the same deletion found in the previously described KOS/29 virus (15), which was constructed by deletion of the PstI fragment from KOS/M strain. For construction of KOS\textsubscript{Pst}LAT, the PstI fragment was deleted from plasmid pRFS (29) to give the pRFS\textsubscript{Pst} plasmid, which was linearized and cotransfected with WT KOS DNA into Vero cells. Progeny viruses were plaque purified and screened by Southern blot analysis for introduction of the PstI deletion into both copies of the HSV-1 LAT loci (data not shown). The KOS\textsubscript{Pst}R rescued virus was constructed by cotransfection of linearized pRFS with KOS\textsubscript{Pst}LAT DNA. Progeny viruses were plaque purified and screened by Southern blot analysis for restoration of the deleted LAT sequences.

**Mouse infections.** Six-week-old male CD-1 mice (Charles River Laboratories) were anesthetized by intraperitoneal injection of ketamine hydrochloride (3.4 mg) and xylazine hydrochloride (0.5 mg) and inoculated with 2 × 10^6 PFU/cell of virus (in a 5-μl volume) onto scarified cornes, as described previously (50). Mice were housed in accordance with institutional and National Institutes of Health guidelines on the care and use of animals in research, and all procedures were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

**ChIP.** Chromatin was prepared from trigeminal ganglia (TG) as previously described with modifications (55). TG were removed from mice at least 28 days postinfection and placed immediately onto dry ice. TG were pooled and mechanically homogenized in Dulbecco’s modified Eagle’s medium to yield cell suspensions. Between four and six TG were used per ChIP assay. Following cell lysis, the chromatin was sonicated to yield DNA fragments of between 150 and 400 bp in length. Immunoprecipitations were carried out as described previously (11). The primers used were as follows: H3K4me3 forward, 5′-GGGGTGGTGCTC-3′; H3K9me3 forward, 5′-GAGT-3′; mouse GAPDH reverse, 5′-CCAGCCAAT CCGTGTCGG-3′; mouse GAPDH forward, 5′-CTGACGTCGCCGCTTGGAA GAA C-3′; and mouse GAPDH reverse, 5′-CCCGCATCGAAGGGTGGAA GAGT-3′. Relative levels of LAT expression were determined by normalizing first to the relative copy number of GAPDH and then to RNA extracted from a KOS\textsubscript{Pst}LAT-infected mouse.

**RESULTS**

**Histone modifications indicative of facultative heterochromatin are associated with viral lytic promoters during latent infection.** We had shown that the HSV lytic gene promoters are enriched for histones containing the H3K9me2 modification during latent infection (55). The goal of this study was to further define the types of heterochromatin on lytic promoters and to investigate the mechanism by which heterochromatin forms on lytic promoters. To this end, we infected mice with WT HSV-1 and performed ChIP on latently infected TG. Immunoprecipitations were carried out using antibodies specific for H3K9me2, H3K9me3, H3K27me3, or H4K20me3. The fraction of DNA immunoprecipitated with each antibody compared to the input was determined by real-time PCR. The levels of heterochromatin on two immediate-early gene (ICP4 and ICP27) promoters along with an early gene (ICP8) promoter were investigated.

In agreement with previous findings, we detected the H3K9me2 modification on all three viral promoters tested, at levels that were enriched above the nonspecific antibody (rabbit immunoglobulin G) control (Fig. 1A to C). In addition, both H3K9me3 and H3K27me3 modifications were enriched on all three viral promoters tested. However, little viral DNA immunoprecipitated with an H4K20me3-specific antibody as compared to the nonspecific antibody control, suggesting that this modification was not present on chromatin associated with the viral promoters tested. By comparison with the cellular GAPDH control, the relative fraction of viral DNA associated with H3K27me3 was increased compared to H3K9me2 and H3K9me3 on all three viral promoters tested (Fig. 1D to F), arguing that higher levels of H3K27me3 than H3K9me2 and H3K9me3 were associated with lytic promoters.

To ensure that the lack of immunoprecipitation of HSV-1 promoter sequences by the antibody specific for H4K20me3 was truly due to lack of the H4K20me3 modification, we examined the chromatin associated with cellular centromere sequences, known to be associated with evHC (43). Analysis of the cellular DNA immunoprecipitated with the various antibodies demonstrated that the H3K9me2, H3K9me3, and H4K20me3 modifications were enriched at the centromeric sequences, but there was little to no H3K27me3 compared to the no-antibody

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control (Fig. 2A). In comparison to the fraction of GAPDH DNA immunoprecipitated, both H3K9me3 and H4K20me3 were highly enriched in centromeric heterochromatin, indicating that the antibodies used were capable of immunoprecipitating cHC (Fig. 2B). Given the low level of enrichment of H3K27me3 and high level of H4K20me3 on centromeric heterochromatin, the chromatin composition of the latent HSV-1 genome did not resemble cellular centromeric heterochromatin.

**Histone modifications associated with the LAT transcriptional unit during latent infection.** Previous studies had indicated that within the LAT transcriptional unit, the promoter and the 5' exon/enhancer are associated with histones bearing markers of euchromatin, namely acetylated histone H3 lysines 9 and 18, with the 5' exon/enhancer having the highest level of enrichment (26, 27). A region downstream of the 5' exon within the region encoding the LAT intron termed CTRL2 (Fig. 3A) had been shown to be bound by the insulator protein CTCF during latency and to possess insulator activities in vitro (1). Because little is known about the heterochromatin in the LAT region, we were interested to determine whether heterochromatin could be detected on specific regions of the LAT transcriptional unit (Fig. 3A).

To investigate the levels and types of heterochromatin on the LAT promoter, 5' exon/enhancer, and a region downstream of the CTRL2 insulator (Fig. 3C), we performed ChIP analysis on TG isolated from mice latently infected with HSV-1. As observed for the lytic genes, H3K9me2, H3K9me3, and H3K27me3 were all enriched above the no-antibody control on the region downstream of CTRL2, whereas H4K20me3 was not enriched above the no-antibody control (data not shown). In comparison to the levels present on the GAPDH promoter, the H3K27me3 modifications were detected to the highest level (Fig. 3D). Lower levels of heterochromatin markers were detected on the LAT 5' exon/enhancer, which is consistent with the previous observation that this region is enriched for acetylated histone H3 during latency (26, 27). The LAT promoter region was associated with histones bearing the H3K27me3 modification characteristic of fHC.

The presence of histone modifications associated with het-
erochromatin on the LAT promoter was surprising, as previous studies had indicated that the LAT promoter is enriched for modifications associated with euchromatin, albeit to lower levels than the 5′/H11032 exon (26, 27). Therefore, we investigated whether we could also detect markers of euchromatin on the LAT promoter at higher levels than a lytic promoter (ICP4).

Because modifications associated with euchromatin had been found at the highest levels downstream of the LAT transcriptional start site, we also investigated a comparable region downstream of the ICP4 gene start site. ChIP analysis was carried out using an antibody against H3K4me3, a marker of both active and poised gene expression (35), and the relative enrichment of viral DNA was normalized to the enrichment of cellular GAPDH DNA. The GAPDH promoter was not enriched for the H3K4me3 modification compared to the nonspecific antibody control in either chromatin isolated from TG or chromatin prepared from HeLa cells (data not shown). Therefore, the enrichment of all viral DNA was either similar to or higher than that for GAPDH. H3K4me3 was enriched on the 5′ exon/enhancer (Fig. 4A), consistent with previous ob-

FIG. 3. Histone modifications in chromatin on the HSV-1 LAT locus during latent infection. (A) HSV-1 genomic map with an expanded view of the long and short internal repeats. The locations of the LAT promoter (P), the 5′ exon/enhancer (exon), and the insulator region (CTRL2) are shown. Also indicated are the 8.3-kb LAT primary transcript and the two major LAT introns of 2 kb and 1.45 kb in length, along with the ICP0 and ICP4 gene transcripts. U, unique short region; U, unique long region. (B) Regions deleted from KOS/PstLAT and KdlLAT viral genomes. (C) Locations analyzed by real-time PCR: P, LAT promoter; 5, LAT 5′ exon/enhancer; C, downstream of CTRL2 (DS CTRL2). (D) ChIP analyses with antibodies specific for H3K9me2, H3K9me3, H3K27me3, and H4K20me3 on TG tissue from mice latently infected with HSV-1. The fraction of viral DNA immunoprecipitated was normalized to the fraction of GAPDH immunoprecipitated. The means and standard errors of the means from at least four independent ChIP assays from three independent infections are shown.

FIG. 4. Association of H3K4me3 with the HSV-1 LAT transcriptional unit and the ICP4 gene promoter during latency. ChIP analyses were conducted using TG tissue from mice latently infected with HSV-1. The fraction of viral DNA immunoprecipitated with the relevant antibody was compared to the input value and normalized to the fraction of cellular GAPDH DNA immunoprecipitated in the same reaction. Primers were specific for the LAT promoter, 5′ exon/enhancer, and region downstream of CTRL2 (DS CTRL2) (A) and the region upstream of the ICP4 gene transcriptional start site (US), across the ICP4 transcriptional start site (SS), and downstream of the ICP4 transcriptional start site (DS) (B). The means and standard errors of the means from three independent ChIP assays from two independent infections are shown.
servations (26, 27). H3K4me3 was also enriched on the LAT promoter and region downstream of the CTRL2 insulator, although at lower levels than on the 5′ exon/enhancer (~5-fold and 1.7-fold lower, respectively) (Fig. 4A). Levels of H3K4me3 on the ICP4 promoter, start site and 5′ untranslated region were lower than those in the LAT region (~20-, 10-, and 30-fold, respectively) (Fig. 4B), and the levels of ICP4 DNA immunoprecipitated were similar to those immunoprecipitated with a nonspecific antibody control (data not shown). Therefore, we could detect the H3K4me3 modifications on the LAT promoter at higher levels than on the ICP4 gene promoter. Hence, it appears that both H3K27me3 and H3K4me3 associate with the LAT promoter.

Levels of H3K9me3 and H3K27me3 on lytic promoters are decreased in the absence of the LATS. We had shown a role for the LATS in promoting heterochromatin formation on lytic promoters in the form of H3K9me2 (55). To investigate whether the LATS play a role in promoting specific histone modifications on lytic promoters during latency, we conducted ChIP analysis on TG isolated from mice latently infected with a LAT deletion virus (KdlLAT) (Fig. 3B) or with the rescued virus (KFSLAT). The KdlLAT virus contains a 1.8-kbp deletion encompassing the LAT promoter, 5′ exon, and 947 bp of the stable LAT intron (Fig. 3B) (20, 29). KFSLAT has the deletion replaced with WT sequences. Consistent with the results with WT virus-infected mice, H4K20me3 was not associated with the lytic promoters that we tested; furthermore, the absence of the LAT had no effect on the levels of H4K20me3 (Fig. 5). In contrast, the levels of the H3K9me3 modification were decreased on all viral promoters tested in TG from mice infected with KdlLAT compared to KFSLAT (ICP4, P = 0.04; ICP27, P = 0.03; ICP8, P = 0.03 [Wilcoxon’s signed-rank test]). Combining the results for all the promoters tested, we observed an average of 1.8-fold more viral DNA associated with H3K9me3 on the KFSLAT genome than on the KdlLAT genome.

Levels of H3K27me3 were also decreased on all of the promoters tested in ganglia from mice infected with KdlLAT virus compared with ganglia infected with KFSLAT virus (Fig. 5) (ICP4, P = 0.03; ICP27, P = 0.03; ICP8, P = 0.03 [Wilcoxon’s signed-rank test]). When results from all of the promoters tested were combined, 3.4-fold higher levels of KFSLAT DNA were associated with H3K27me3 than with KdlLAT. In total, these results argued that the relative fraction of lytic gene promoters associated with H3K27me3 was decreased more than that associated with H3K9me3 following infection with the LAT-null virus.

Analysis of a LAT promoter deletion mutant virus. Our previous analyses had utilized a LAT deletion mutant virus lacking the promoter, 5′ exon, and part of the intron (55). To test a mutant virus with a more specific effect on LAT expression, we constructed a LAT promoter deletion mutant virus, as described in Materials and Methods, by deleting a 202-bp fragment containing the core LAT promoter from HSV-1 to produce the KOSΔPstLAT virus (Fig. 3B). The corresponding rescued virus was also constructed by restoration of the deleted sequences into KOSΔPstLAT to give the PstRLAT virus.

To determine the effect of the LAT promoter deletion on LAT expression, we infected mice with KOSΔPstLAT or PstRLAT virus and measured LAT expression by real-time RT-PCR carried out on RNA from TG isolated from latently infected mice. We observed an ~1,000-fold decrease in the levels of LAT detected within latently infected TG of mice infected with KOSΔPstLAT compared to PstRLAT (Fig. 6A), confirming that the mutant virus was indeed defective for LAT expression during latency. The decreased expression of the LAT from KOSΔPstLAT was not a result of a decreased latent DNA load, as there was an ~3.7-fold increase in the relative levels of DNA isolated from mice latently infected with KOSΔPstLAT compared to those latently infected with the PstRLAT virus (Fig. 6B).

We then tested the effect of the mutation on chromatin association with viral lytic gene promoters by ChIP analysis. In ganglia from mice infected with KOSΔPstLAT, we observed a statistically significant decrease in the fraction of ICP4 and ICP27 promoter DNA sequences associated with H3K27me3 during latency, as compared with WT of mice infected with PstRLAT virus (Fig. 6C and D) (P < 0.05 by Wilcoxon’s signed-rank test). Although there were slightly decreased levels of viral DNA associated with H3K9me2 and H3K9me3 following infection with KOSΔPstLAT, these differences were not statistically significant (P > 0.05). Therefore, specific de-
letion of the LAT promoter, and hence a reduction in LAT expression, resulted in decreased levels of H3K27me3 on viral lytic gene promoters.

**DISCUSSION**

We and others had shown previously that mutation of the HSV-1 LAT gene results in elevated lytic gene expression during acute (20) and latent (9) infection and reduced levels of the heterochromatin marker H3K9me2 (55). In this study, we further defined the structure of the latent viral chromatin by examining the heterochromatin markers on the HSV-1 genome during latency and found that the major heterochromatin modification on the histones was associated with HSV-1 lytic gene promoters is H3K27me3, a marker for cellular fHC. Furthermore, H3K27me3 is reduced on viral lytic gene promoters in ganglionic tissue latently infected with a LAT mutant virus missing the LAT promoter, 5'/H11032 exon, and part of the intron or with a viral mutant lacking only the LAT promoter. Thus, the lack of LAT transcription is the likely cause of the reduced H3K27me3, thus pointing to a possible mechanism for RNA silencing.

**fHC is associated with HSV-1 lytic promoters during latent infection.** We observed that viral lytic gene promoters are enriched for the H3K9me2/3 and H3K27me3, but not H4K20me3, histone modifications during latent infection. In contrast, cellular centromeric heterochromatin is enriched for the H3K9me3 and H4K20me3, but not H3K27me3, histone modifications. Therefore, the chromatin modifications present on HSV-1 lytic promoters during latency do not resemble cellular centromeric heterochromatin. Because H3K27me3 is found at high levels within regions of fHC and not within cHC, these results indicate that heterochromatin similar to cellular fHC is associated with lytic gene promoters during latency.

fHC is defined by its ability to be converted into euchromatin to allow active gene expression. Because HSV-1 must overcome gene silencing for reactivation, it makes sense that viral lytic promoters on at least a portion of viral genomes are assembled into chromatin resembling cellular fHC and not the more stable cHC. However, given the recent discoveries of histone demethylases that are able to remove modifications associated with cHC (22, 57), it was also possible that the genome was associated with cHC. In addition to H3K27me3, the H3K9me2/3 modifications are also associated with lytic gene promoters, albeit to lower levels than H3K27me3. Although H3K9me3 is classically associated with cHC, it has also been detected within regions of fHC such as the inactive X chromosome (6, 41). Therefore, it is possible that H3K9me3 is present on histones associated with genomes also bearing the H3K27me3 modification. In the case of HIV latency, the latent viral genome has been found to be associated with H3K9me3 and the heterochromatin HP1 (18, 33). A recent study also found H3K27me3 to be associated with the latent HIV genome (38), suggesting a common pattern of histone modifications on latent viral genomes. However, a further possibility is that the two modifications are found on different HSV-1 genomes and potentially different populations of latently infected neurons. Given that populations of neurons have been previously characterized that are positive for HSV-1 DNA but do not express detectable levels of the LAT (10, 34, 39, 42), it is possible that

![FIG. 6. Comparison of histone modifications in chromatin on HSV-1 promoters during latent infection with KOSΔPstLAT or PstRLAT viruses. (A) Relative LAT expression in KOSΔPstLAT and PstRLAT-infected TG as determined by real-time RT-PCR analysis. The relative quantity of LAT cDNA compared to GAPDH cDNA levels was normalized to a KOSΔPstLAT-infected sample. (B) Relative quantity of viral DNA isolated from the TG of mice latently infected with KOSΔPstLAT and PstRLAT. Real-time PCRs were carried out on input DNA from the ChIP assays using primers specific for the ICP4 and cellular GAPDH promoters. The relative amount of viral DNA was normalized first to the amount of cellular DNA and then to the relative amount of viral DNA in the PstRLAT samples. ChIP analyses were conducted with antibodies specific for H3K9me3, H3K27me3, or H4K20me3 (C and D). The fraction of ICP4 (C) and ICP27 (D) promoter DNA was normalized to the fraction of GAPDH DNA immunoprecipitated in the same reaction. The means and standard errors of the means from at least four independent infections are shown. Samples with values that vary significantly (P < 0.05 by Wilcoxon’s signed-rank test) are indicated by asterisks.]
different neuronal populations exhibit different types of heterochromatin.

Histone modifications on the LAT transcriptional unit during latency. The HSV-1 LAT transcriptional unit is the only region of the viral genome that undergoes active transcription during latent infection. Previous studies found that the LAT promoter and 5′ exon/enhancer region were associated with acetylated histones, a hallmark of euchromatin, with the 5′ exon/enhancer exhibiting an approximately threefold increase in acetylated histones compared to the promoter (26, 27). Consistent with its role in maintaining an open chromatin conformation, we also found that the 5′ exon/enhancer was the only region of the viral genome examined that is not enriched for histone modifications associated with heterochromatin during latency.

Our observation that the LAT promoter is associated with H3K9me3 and H3K27me3 was perhaps surprising given that transcription initiates from the LAT promoter. One possibility is that the dual presence of H3K9me3 and H3K27me3 represents genomes not undergoing active transcription of the LATs. Indeed, a number of studies have found that a large proportion of latently infected neurons do not express detectable levels of the LATs (10, 34, 39, 42). However, this would imply that even in the absence of active transcription, the 5′ exon/enhancer does not associate with heterochromatin, whereas the promoter would associate with heterochromatin.

A further possibility is that transcription of the LAT occurs in the presence of heterochromatin on the promoter and is perhaps required for transcription. The LAT promoter appears to be in a bivalent chromatin state, containing markers of both heterochromatin and euchromatin. There is evidence that RNA polymerase II can be recruited to regions containing both the H3K27me3 and H3K4me3 modifications but is blocked from elongating due to the presence of heterochromatin on 5′ untranslated regions (47). The presence of euchromatin on the LAT 5′ exon may therefore allow RNA polymerase II elongation to occur even though H3K27me3 is present on the LAT promoter and would further reinforce the critical role of the 5′ exon in maintaining long-term expression of the LATs (5, 30). Furthermore, a requirement of H3K27me3 formation on the Xist promoter during the initiation of X chromosome inactivation has previously been found (48) and may therefore be a common requirement of noncoding RNA transcription in the formation of heterochromatin.

The 5′ exon/enhancer is separated from nearby lytic promoters by the presence of an insulator element known as CTRL2 (1). CTRL2 has been identified as an insulator based on the binding of the mammalian insulator protein, CTCF, and its enhancer-blocking activity in vitro (1, 8). Although CTFC has been found to exhibit enhancer-blocking activity in mammalian cells (3), until recently there was no direct evidence for CTFC in separating active and repressive domains (12). Our data on the association of heterochromatin with the LAT region further reinforce a potential role for CTCF binding in forming a barrier between the LAT 5′ exon/enhancer and a region downstream of CTRL2 and hence the ICP0 coding region. However, a modification associated with euchromatin, H3K4me3, was detected downstream of CTRL2. Therefore, our data on the histone modifications on surrounding CTRL2 suggest that it functions to prevent the spreading of heterochromatin from lytic genes onto the LAT 5′ exon/enhancer but may not confine euchromatin to the LAT 5′ exon/enhancer.

Comparison of heterochromatin on the KdILAT and KOSAPstLAT genomes. Our previous study found a role for the LATs in promoting H3K9me2 and preventing the euchromatic modification, H3K4me2, on lytic promoters during latency (55). Here we extend those results to conclude that the LAT specifically promotes the formation of fHC. The virus used by Wang et al. was KdILAT, which contains a 1.8-kb deletion encompassing the LAT promoter, 5′ exon, and a portion of the CTRL2 insulator (20, 29). Therefore, although the levels of H3K9me2 (55), H3K9me3, and H3K27me3 were decreased following infection with KdILAT compared to KFS-LAT, it was possible that deletion of cis-acting DNA sequences and not reduced LAT expression resulted in the reduced heterochromatin formation phenotype. To rule out the possibility that deletion of the 5′ exon/enhancer and the insulator caused the decreased heterochromatin on lytic promoters following infection with KdILAT, we constructed a LAT promoter deletion virus, KOSAPstLAT. From our experiments with KOSAPstLAT, we have identified a clear role for the LATs in promoting H3K27me3 formation on lytic promoters during latency. These results provide the strongest connection to date between LAT transcription and heterochromatin on viral lytic gene promoters.

Additional sequences deleted from the KdILAT genome but not the KOSAPstLAT genome may have affected the levels of H3K9me2/3 on lytic promoters. The deletion in KdILAT encompasses the 5′ exon and a portion of the CTRL2 insulator. It seems unlikely that deletion of the 5′ exon resulted in the increase in H3K9me2/3 formation on lytic promoters as this is the only region of the genome that we found to be depleted of markers associated with heterochromatin and has been found to be enriched for markers of euchromatin (26). Although we failed to detect clear insulator function for CTRL2 in confining euchromatin to the 5′ exon/enhancer, it is still possible that deletion of a portion of the CTRL2 insulator resulted in decreased H3K9me3 formation on lytic promoters. Furthermore, insulators can function to mediate long-range genomic interactions (32, 45, 58). Hence, deletion of a portion of CTRL2 could disrupt any long-range interactions within the HSV-1 genome to reduce heterochromatin association.

Role for LAT noncoding RNA in promoting fHC formation. Although potential open reading frames have been detected within the LAT transcriptional unit (7, 51), no LAT-encoded protein product has been reliably detected within latently infected neurons (16, 17). Furthermore, the nucleotide sequence of the LAT region of different HSV-1 strains has been reported to be more highly conserved than the predicted amino acid sequences (7). Therefore, it is likely that the LATs function as noncoding RNAs, in this case, to promote fHC on the viral genome. Noncoding RNAs are emerging as cofactors in the targeting of fHC formation. In the case of the inactive X chromosome, the Xist RNA has been known for some time to play a role in the induction of gene silencing (37). Recently an additional RNA also encoded from the Xist locus, termed RepA, was identified (60). RepA has been found to interact with the polycomb repressor complex 2 (PRC2), which contains a histone methyltransferase responsible for the H3K27me3 modification, and target it to the inactive X chromosome.
RepA is not unique among the noncoding RNAs in binding to PRC2. Noncoding RNAs have been identified that are required for fHC formation on the Hox D locus (HOTAIR) (40) and the Kcnq1 imprinted domain (Kcnq1ot1) (36). Both the HOTAIR and Kcnq1ot1 RNAs have been found to interact with PRC2 to target it to DNA. Recruitment of PRC2 through an interaction with RNA may explain why no DNA-binding subunit has been identified within mammalian PRC2. If RNA binding is the key mechanism utilized by PRC2 to target it to DNA, then it is possible that though interacting with the LAT RNA, PRC2 is targeted to viral promoters to result in H3K27me3 methylation.

To date, the noncoding RNAs identified as interacting with PRC2 to target it to chromatin are all over 2 kb in length (36, 40, 60), which would be in keeping with a role for the LAT intron or primary transcript in the initiation of fHC formation. Nevertheless, a role for the microRNAs within the LAT region cannot be ruled out, especially given that an endogenously expressed mammalian microRNA capable of targeting H3K27me3 has recently been identified (21); however, the binding of PRC2 to microRNAs has not been investigated. Regardless of the nature of the exact RNA species within the LAT region required for the fHC target, these results provide further evidence of the role of RNAs of both mammalian and viral origin as cofactors for fHC formation.

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ADDITION IN PROOF

We learned of the article by Kwiatkowski et al. (D. L. Kwiatkowski, H. W. Thompson, and D. C. Bloom, J. Virol. 83: 8173–8181, 2009) upon its electronic publication. Our conclusions are similar to those of Kwiatkowski et al. in that they conclude that “expression of the LAT during latency may act to promote an appropriate heterochromatic state that represses lytic genes but is still poised for reactivation.” Our experimental results do differ somewhat in that Kwiatkowski et al. report that the HSV-1 17ΔPst mutant virus shows higher levels of H3K27me3 than those seen with wild-type 17syn+, in contrast with our results in which HSV-1 KOSΔPstLAT shows reduced levels of H3K27me3 compared with those seen with the rescued virus PstLat. Possible explanations for the differences in the results from the two articles include the following. (i) Different virus strains were used. (ii) Different animal models were used. (iii) We compared the mutant strains with rescued viruses, while Kwiatkowski et al. compared the mutant strain to the wild-type parent. (iv) We compared mutant and rescued virus infections in parallel within the same experiment, while Kwiatkowski et al. compared the wild-type and mutant virus infection data from separate experiments.

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